Supplementary Materials

T cells expressing CD19/CD20 bi-specific chimeric antigen receptors prevent antigen escape by malignant B cells

Eugenia Zah, Meng-Yin Lin, Anne Silva-Benedict, Michael C. Jensen, Yvonne Y. Chen

Supplementary Materials and Methods

Plasmid Construction

Bi-specific CD19-CD20 CARs were constructed by isothermal assembly (1) of DNA fragments encoding the CD19 scFv derived from the FMC63 monoclonal antibody (mAb) (2), the CD20 scFv derived from the Leu-16 mAb (3), an IgG4-derived extracellular spacer, as well as the cytoplasmic domain of 4-1BB and CD3 zeta chain. Sequences of the extracellular spacer and linker sequences connecting scFv domains are listed in Supplementary Table S1. All CARs were fused to a truncated epidermal growth factor receptor (EGFRt) via a T2A peptide to facilitate antibody staining and sorting of CAR-expressing cells (4). CAR constructs were cloned into the epHIV7 plasmid backbone (5) for packaging into third-generation, self-inactivating lentiviruses. To generate CD19+ and CD20+ K562 cell lines, CD19 and CD20 messenger RNA were isolated from TM-LCL cells using GenElute mRNA Miniprep kits (Sigma-Aldrich) and then reverse-transcribed using SuperScriptIII First-Strand Synthesis System (Life Technologies) following manufacturer's protocols. CD19 was PCR amplified with forward primer 5' AATAAAGCTAGCATGCCACCTCCTCGC and reverse primer 5' AATAAAGCGGCCGCTTA TCTTTTCCTCCTCAGGACCAG; CD20 was PCR amplified with forward primer 5' AATAGCTAGCATGACAACACCCAGAAATTCAGTAAA and reverse primer 5' AATAGCGGCCGCTTAAGGAGAGCTGTCATTTTCTATTGG. Both genes were

inserted into epHIV7 via NheI and NotI sites (underlined), packaged into lentiviruses, and stably integrated into K562 cells. For gene editing of Raji cells by clustered, regularly interspaced, short palindromic repeat (CRISPR) technology (6), a guide RNA (gRNA) sequence targeting exon 1 of the CD19 gene was identified using the online CRISPR design tool (http://tools.genome-engineering.org). Forward (5' CACC **G** *CGAGGAACCTCTAGTGGTGA*) and reverse (5' AAAC *TCACCACTAGAGGTTCCTCG* **C**) oligonucleotides containing the gRNA sequence (italicized) and an additional 'G' (bolded) inserted for efficient U6 promoter transcription were annealed to each other in annealing buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1mM EDTA) at 95°C for 4 min and cooled at room temperature for 15 min. The annealed oligos were inserted into the pX330 plasmid (Addgene) (7) at the BbsI site to generate the CD19-CRISPR plasmid. The neomycin resistance gene was subsequently linked via a T2A sequence to the 3' end of the Cas9 gene in a two-step cloning procedure to generate CD19-CRISPR-T2A-NM. T2A was cloned into the CD19-CRISPR plasmid via the FseI and EcoRI sites while introducing an AvrII site at the 3' end of T2A upstream of EcoRI. The neomycin resistance gene was PCR amplified from a pcDNA3.1(+)-based plasmid (forward primer: 5' TTATCCTAGGATGATTGAACAAGATGGATTGCACG; reverse primer: 5'TTATGAATTCTCAGAAGAACTCGTCAAGAAGG) and inserted via AvrII and EcoRI sites (underlined). All oligonucleotides were synthesized by Integrated DNA Technologies and constructs were sequence verified by Retrogen Inc.

Generation of CAR-expressing primary human T cells

CD8+ T cells were isolated from healthy donor whole blood obtained from the UCLA Blood and Platelet Center. Whole blood was treated with RosetteSep CD8+ T-cell enrichment cocktail

(Stem Cell Technologies), and CD8+ T cells were then isolated by Ficoll-Paque density-gradient separation (GE Healthcare). For central-memory T (T_{CM}) cell isolation, CD8+ T cells were depleted of CD45RA+ cells and enriched for CD62L+ expression using magnetic bead-based isolation (Miltenyi). Isolated T cells were immediately stimulated with CD3/CD28 T-cell activation Dynabeads (Life Technologies) at a 1:1 bead:cell ratio. Three days post-isolation, T cells were transduced with concentrated lentiviral supernatant (MOI = 1.5) supplemented with 5 μg/mL polybrene (Sigma-Aldrich) and centrifuged for 30 min at 800 xg at room temperature. T cells were expanded in complete T-cell medium supplemented with 100 U/mL penicillin-streptomycin (Life Technologies). Cultures were also fed with 50 U/mL IL-2 (Life Technologies) and 1 ng/mL IL-15 (Miltenyi) every 48 hours. Dynabeads were removed 9 or 10 days post-isolation. CAR+ cells were enriched by staining transduced T cells with biotinylated Erbitux (Bristol-Myers Squibb, biotinylated in house) followed by anti-biotin–conjugated microbeads (Miltenyi) and sorted by magnetic separation. Immediately after cell sorting, CAR+ T cells were expanded by stimulation with irradiated (8,000 rad) TM-LCLs at a T cell:TM-LCL ratio of 1:7, with 1 x 10^6 T cells in 50 mL complete media per T75 culture flask. Mock-transduced T cells were stimulated using the rapid expansion protocol as previously described (8). Expanding cells were cultured in complete medium supplemented with 50 U/mL IL-2 and 1 ng/mL IL-15 every 48 hours.

Lentivirus preparation

HEK 293T cells seeded in 10-cm dishes at 3.5 x 10^6 cells in 9 mL DMEM +10% HI-FBS media were transfected by calcium phosphate following manufacturer's protocol (Clontech). Sixteen hours post-transfection, cells were washed with 5 mL of 1X-phosphate buffered saline without

magnesium and calcium (PBS) (Lonza) and supplemented with fresh media containing 60 mM sodium butyrate (Sigma-Aldrich). Viral supernatant was collected 24 hours and 48 hours after the media change, and cell debris was removed from the supernatant by centrifugation at 10,000 rpm for 10 min at 4°C, followed by filtration through a 0.45 μM membrane (Corning). Viral supernatant collected 24 hours after media change was mixed with ¼ volume 40% polyethylene glycol 8000 (PEG) (Amresco) in 1X-PBS and rotated overnight at 4°C. PEG-treated virus was pelleted at 3,000 rpm for 20 min at 4°C, then resuspended in viral supernatant collected 48 hours after media change, and finally ultracentrifuged at 24,500 rpm for 1 hour and 35 minutes at 4°C. Pellets were resuspended in 200 μL of serum-free RPMI-1640 and then incubated for 1 hour at 4°C to allow complete dissolution. Virus was then stored at -80°C for subsequent titer and use.

Flow cytometry. T-cell subtype, target antigen expression, and activation marker expression were characterized by surface staining with fluorophore-conjugated antibodies for CD3, CD8, CCR7, CD45RA, CD62L, CD19, CD20, CD69, CD107a, CD137, Lag-3, Tim-3, and PD-1 (BD Biosciences, BioLegend, and Miltenyi). CAR surface expression was measured directly by staining with Protein L (Genscript) or indirectly by staining EGFRt with biotinylated Erbitux (Chiron; biotinylated in-house) followed by PE-conjugated streptavidin (Jackson ImmunoResearch). Flow cytometry was performed on a MACSQuant VYB instrument (Miltenyi), and data were analyzed using FlowJo cell analysis software (TreeStar).

Western blots

Two million cells were harvested. For CAR expression analysis, harvested cells were treated with 1 mg/mL tunicamycin (MP Biomedicals) overnight. Cells were subsequently lysed in 30

μL of lysis buffer containing RIPA buffer (1% Igepal CA-630, 0.1% SDS, 0.5% sodium deoxycholate, with protease and phosphatase inhibitor) supplemented with 0.5 mM phenylmethanesulfonylfluoride (PMSF) for 45 min on ice. Centrifuged cell lysate was collected and mixed with Bolt LDS sample buffer (Life Technologies) containing 5% β-mercaptoethanol (Sigma-Aldrich) and heated at 70°C for 10 min. Protein separation was performed by SDS-PAGE with pre-cast 4-12% Bis-tris gels (Life Technologies). Gels were transferred to nitrocellulose membranes using the iBlot gel transfer system (Invitrogen). Membranes were blocked with 0.5% bovine serum albumin (VWR) or 5% nonfat milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 2 hours and then probed with antibodies either overnight or using the SNAP i.d. 2.0 Protein Detection system (Millipore). Primary antibodies for CD19 (eBioscience), CD3 zeta (BD Biosciences), and GAPDH (Sigma-Aldrich), as well as HRP-conjugated anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch) were used. Membranes were treated with SuperSignal West Dura Extended Substrate (Thermo Scientific) and imaged using the ChemiDoc XRS+ System (Bio-Rad). Gel images were quantified using the ImageJ software.

Cytotoxicity assay

Target cells (K562 cells) seeded at 1x104 cells/well in a 96-well plate were co-incubated with effector cells at varying effector to target (E:T) ratios in complete media without phenol red and with 5% HI-FBS for 4 hours. Supernatants were harvested and analyzed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). Colorimetric changes were assayed using the Eon Microplate Spectrophotometer (BioTek) at 490 nm and data were collected using the

Gen5 data analysis software (BioTek). Calculations for % cell lysis were performed following the CytoTox 96 kit manual using the equation:

$$\%\ \text{Cytotoxicity (Lysis)} = \frac{Experimental - Effector\ Spontaneous - Target\ Spontaneous}{Target\ Maximum - Target\ Spontaneous} \times 100$$

where italicized terms refer to Abs490 readings of the corresponding samples.

Cytokine production quantification

Target cells were seeded at 5 x 10^4 cells/well in a 96-well plate and co-incubated with effector cells at an E:T ratio of 2:1 for 24 hours. IFN-γ, TNF-α, IL-2 , IL-4, IL-6, and IL-10 levels in the culture supernatant were measured with the BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit II (BD Biosciences). Results were collected by flow cytometry on a MACSQuant VYB instrument and analyzed using the FCAP Array v3.0.1 software (BD Biosciences).

***In vivo* xenograft studies in NOD/SCID/γc-/- (NSG) mice**

All *in vivo* experiments were approved by the UCLA Institutional Animal Care and Use Committee. Six- to eight- week old female NSG mice were bred in-house by the UCLA Department of Radiation and Oncology. Half a million EGFP+, firefly luciferase (ffLuc)-expressing Raji cells (either wildtype (WT) alone or a mixture of WT and CD19– knockout cells at a WT:knockout ratio of 3:1) were administered to NSG mice via tail-vein injection. Seven days later, engrafted target cells were treated with 10 x 10^6 mock-transduced or CAR+/EGFRt+ cells via tail-vein injection. For bioluminescence imaging, mice were anesthetized with isofluorane, injected intraperitoneally with 3 mg D-luciferin (Gold Biotechnology) in 100 µl PBS, and imaged 10 min later using an IVIS Lumina III LT Imaging System (Perkin Elmer) at an acquisition time of 1 sec to 1 min. Photon flux was analyzed using Living Image Software

(Perkin Elmer). Peripheral blood was obtained by retro-orbital bleeding 10 days and 20 days post tumor-cell injection. Prior to staining, red blood cells were lysed using red blood cell lysis solution (Miltenyi). Bone marrow from hind-leg tibias and femurs was collected at the time of animal sacrifice. Bone marrow was washed from the bones with 2 mM EDTA 0.5% BSA PBS and treated with 0.2% NaCl solution followed by 1.6% NaCl solution to lyse red blood cells found in the marrow. Blood and bone marrow samples were analyzed by flow cytometry as described above. Tumor cells were identified by EGFP expression; T cells were identified by CD8+/EGFRt+ phenotype.

Supplementary Materials References

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Supplementary Figure Legends

Fig. S1. CD20 CAR T cells with a long extracellular spacer demonstrate superior *in vitro* functionality. A, design of CD20-specific CARs containing long, medium, and short extracellular spacers. Hinge, CH2, and CH3 are domains within human IgG4. B, verification of CAR expression by EGFRt staining of lentivirally transduced primary CD8+ T cells. C-E, CD20 CAR-T cells were co-incubated with K562 target cells. C, target-cell lysis was measured after a 4-hour co-incubation. D, IFN-γ, TNF-α, IL-2 production was measured (in relative fluorescence units; RFU) after a 24-hour co-incubation. E, T-cell proliferation was measured after a 7-day co-incubation with or without target cells.

Fig. S2. CD19-OR-CD20 CARs are efficiently expressed on the surface of primary human T cells. A, expression of CARs with long and short extracellular spacers on EGFRt-sorted CD8+ T cells was detected by surface staining with protein L, which binds to the scFv domain of the CAR. B, full-length expression of the CARs in (A) was verified by western blot probed with anti-CD3 zeta antibody. C, expression of CARs with short extracellular spacers and various scFv linkers on EGFRt-sorted CD8+ T cells was detected by surface staining with protein L. D, full-length expression of the CARs in (C) was verified by western blot probed with anti-CD3 zeta antibody.

Fig. S3. CRISPR/Cas9-edited Raji cells do not express CD19. A, CD19 surface antibody staining. B, western blot for CD19 in Raji cell lysates.

Fig. S4. Improved CD20 targeting does not compromise CD19 detection by OR-gate CARs. CAR-T cells were co-incubated with WT Raji or CD19+ K562 targets for 24 hours. A, CD69, CD137, and CD107a surface expression was quantified by flow cytometry. B, IFN-γ, TNF-α, and IL-2 production was quantified by multiplex bead-based assay. C, Lysis of CD19+ K562 cells by T cells expressing no CAR (Mock), single-input CARs (CD19 Short and CD20 Long), or various 20-19 Short OR-gate CARs after a 4-hour co-incubation. Reported values are the mean of triplicates, with error bars indicating one SD.

Fig. S5. OR-gate CAR-T cells do not become exhausted earlier or more severely compared to single-input CD20 CAR-T cells. Mock-transduced and CAR-T cells were co-incubated with WT Raji targets for 6 days, and then co-incubated with CD19– mutant Raji targets for another 6 days. (Note: mock-transduced T cells had been overwhelmed by WT Raji by day 6 and were not re-challenged with mutant Raji.) Exhaustion marker staining of CAR-T cells (A) before antigen stimulation (B) 48 hours after co-incubation with WT Raji, and (C) 48 hours or (D) 6 days after subsequent co-incubation with CD19– Raji cells. At each time point, cells were surface-stained for Lag-3, Tim-3, and PD-1. Values shown are the mean of triplicates, with error bars indicating one SD.

Fig. S6. T cells expressing OR-gate CARs, but not single-input CD19 CARs, can efficiently target both WT and CD19– mutant Raji tumors *in vivo*. A, survival of NSG mice bearing purely WT or mixed (75% WT, 25% CD19–) Raji tumor xenografts and treated with T cells expressing no CAR (Mock) or single-input CD19 CAR (CD19 Short). B, femoral bone marrow of mice bearing mixed Raji tumors from (A) was collected at the time of sacrifice and analyzed by flow

cytometry. Raji cells were identified by the expression of EGFP, which was stably integrated into both Raji cell lines, and CD19 staining was performed to distinguish WT and CD19– mutant Raji populations. *P*-value was calculated by two-tailed Student's t-test. C, survival comparison between mice bearing WT or mixed Raji xenografts and treated with OR-gate CAR-T cells. D, survival of mice bearing purely WT Raji tumor xenografts treated with T cells expressing no CAR, the single-input CD19 CAR, or OR-gate CARs. E, frequency of CD8+/EGFRt+ OR-gate CAR-T cells in the peripheral blood 3 days and 13 days after T cell transfer. (Note: mice treated with CD19 CAR-T cells were too ill by day 13 post T-cell injection to permit retro-orbital bleeding.) N = 5 in all test groups. *P*-values for Kaplan-Meier curves were calculated by log-rank test analysis; n.s.: not significant ($p > 0.1$); *: $p < 0.1$.

Fig. S7. Raji cells retain antigen expression *in vivo*. Bone marrow recovered from NSG mice at the time of sacrifice was analyzed for the presence of Raji cells and their CD19 and CD20 expression levels. Raji cells were identified by the expression of EGFP. Each row of plots was generated from the bone marrow of five animals treated with the same CAR–T-cell line, which is indicated above each row. A, samples recovered from animals engrafted with WT Raji tumors. B, samples recovered from animals engrafted with mixed Raji tumors.

Table S1. Primers for cloning of bi-specific CARs

Name	Sequence
IgG4 hinge	GAGAGCAAGTACGGACCGCCCTGCCCCCCTTGCCCT
IgG4 CH2	GCCCCCGAGTTCCTGGGCGGACCCAGCGTGTTCCTGTTCCCCCC CAAGCCCAAGGACACCCTGATGATCAGCCGGACCCCCGAGGTG ACCTGCGTGGTGGTGGACGTGAGCCAGGAAGATCCCGAGGTCC AGTTCAATTGGTACGTGGACGGCGTGGAAGTGCACAACGCCAA GACCAAGCCCAGAGAGGAACAGTTCAACAGCACCTACCGGGTG GTGTCTGTGCTGACCGTGCTGCACCAGGACTGGCTGAACGGCAA AGAATACAAGTGCAAGGTGTCCAACAAGGGCCTGCCCAGCAGC ATCGAAAAGACCATCAGCAAGGCCAAG
IgG4 CH3	GGCCAGCCTCGCGAGCCCCAGGTGTACACCCTGCCTCCCTCCCA GGAAGAGATGACCAAGAACCAGGTGTCCCTGACCTGCCTGGTG AAGGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCA ACGGCCAGCCTGAGAACAACTACAAGACCACCCCTCCCGTGCTG GACAGCGACGGCAGCTTCTTCCTGTACAGCCGGCTGACCGTGGA CAAGAGCCGGTGGCAGGAAGGCAACGTCTTTAGCTGCAGCGTG ATGCACGAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGA GCCTGTCCCTGGGCAAG
Short Flexible (G4S)1	GGA GGT GGT GGA TCC
Long flexible (G4S)4	GGT GGA GGC GGC AGT GGC GGA GGT GGG AGC GGA GGG GGC GGT TCC GGT GGC GGG GGA TCT
Short Rigid (EAAAK)1	GAG GCC GCA GCG AAG
Long Rigid (EAAAK)3	GAA GCC GCC GCA AAG GAA GCT GCT GCC AAA GAA GCA GCC GCT AAG